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Leaf Pigments Help Almond Explants Tolerating Osmotic Stress

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***Abstract*—**This study was conducted to evaluate the response of almond genotypes to osmotic stress *in vitro* in order to screen drought tolerance. Explants subjected to polyethyleneglycol osmotic stress (0, 3.5, and 7.0% WV) on the MS medium. Concentrations of photosynthesis pigments, anthocyanins, and carothenoids were significantly reduced under osmotic stress. Under osmotic stress, leaf water content, cellular membrane stability and pigments concentrations were significantly higher in the leaves of drought tolerant genotypes. The results revealed that carotenoids and anthocyanins may act as photoprotectant compounds in almond leaves and involved in drought tolerance system of the plant.

***Keywords*—**Almond, Anthocianins, Carotenoids, *in vitro*, Leaf Osmotic Stress, Leaf Pigments, Polyethylene Glycol.

I. Introduction

WATER limitation is an important factor to reduce agricultural crop production, which is related to global warming and climate changes. Crops cultivated under Mediterranean climates usually subjected to drought stress during growth season. Almond (*Prunus dulcis* (Mill.) D.A. Webb) is a major nut crop cultivated under Mediterranean climates. Almond originated from central and southwest Asia, and represent divergent evolution under cold and xerophytic environments [1]. Although almond known as a drought tolerant plant, its yield is susceptible to drought stress. Almond produces 1400–1800kg kernel per hectare in irrigated orchards; however, its productions may be reduced to about 800–900kg under drought conditions [2]. Isaakidis et al. [3] stated that almond yield loss under drought stress is probably due to reduced photosynthesis activity of the plant.

Yadollahi et al. [4] showed genetic diversity in drought tolerance of almonds. Hence, screening drought tolerant almond genotypes may help to cope with drought problem in semi-arid and arid condition. However, screening of drought tolerant genotypes is a time consuming, laborious, and costly process. Under such circumstance, *in vitro* experiments have been introduced as good alternative for field experiments to screen drought tolerance in woody species [5]. It is possible to evaluate responses of large number of explants to induced drought stress *in vitro* and screen drought tolerance. Having more control on environmental factors and experimental treatments also have stated as other advantages of *in vitro* experiments [6]. The effects of *in vitro* induced osmotic stress have been reported on many crops, including tobacco [7], carrot [8], alfalfa [9], tomato [10], sunflower [11], rice [12], and common fig [5].

Polyethylene glycol (PEG) is usually used to reduce water potential of media in the experiments. It reduces Osmotic potential in media and simulates the drought condition without exerting any toxic effects or absorption by plants [13], [14]. This study was conducted to evaluate five high yield and late bloom almond genotypes to osmotic stress induced by PEG *in vitro*. Responses of the almond genotypes compared to the drought tolerant almond × peach hybrid, GF677 rootstock, in order to screen drought tolerant genotypes.

II. Material and Methods

The almond genotypes used included ‘Mamaei’, ‘Sepid’, ‘B-124’, ‘Supernova’ and ‘Ferragnès’ and almond×peach hybrid, GF677, which were obtained from the almond collection of Seed and Plant Improvement Institute (SPII), Karaj, Iran. Current season shoots of 4-year-old almond trees were excised 90 days after starting the active growth, in June 25th, 2011. The shoots were placed under running tap water for an hour and submerged in 3% mercury chloride solution for 90 s. Shoots were rinsed three times in sterile distilled water and then explants with 15–20mm length (single node) were prepared.

Explants individually transferred to jars containing 15ml of the Murashige and Skoog (MS) basal medium. The medium were supplemented with 30g/L sucrose, 1g/L benzyl adenine (BA) and 8g/L agar. The pH of the media was adjusted to 5.7 ± 0.05 with HCl 0.1N or NaOH 0.1N prior to sterilization by autoclaving at 121˚C for 15min. Cultures were maintained at 25±3C and 16:8h photoperiod of cool-white light at 1250 Lux. After 30 days, uniform developed explants were excised and transferred to the same medium but containing 0.1 mg/L BAP. The explants were maintained at the same conditions described above for another 30 day period.

Uniform developed explants were selected and transferred to the MS media containing different concentrations of poly ethylene glycol (PEG) namely 0, 3.5%, and 7%. No plant growth regulator was used during this step. The incubation conditions were the same as described above. After 40 days, at the end of the osmotic stress period, leaf water content was saturation deficit (WSD) in the first three fully expanded

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leaves at the top of each explants was determined. Leaves were dried at 70˚C for 48h to determine dry mass. Leaf WSD was calculated using the following formula:

$$WSD = 100\times(SW-FW)/(SW-DW)$$

In this formula SW, FW and DW were the saturated weight, fresh weight and dry weight of leaves, respectively.

Cell membrane stability (CMS) was measured by using the method described by Blum and Ebercon [15]. Photosynthesis pigments were measured in leaf discs with a known area ($10\times50.24mm^2$ discs). The discs were cut into smaller pieces and extracted with 5mL of DMSO at 70ºC for 30min [6]. Absorbance of the extract was measured by a spectrophotometer at 470, 646 and 663nm. Concentration of chlorophyll a and b, the ratio between them, total chlorophyll content and the carotenoids were determined following the equation proposed by Wellburn [16].

Five hundred mg leaf material was homogenized in 1ml of acidified (1% HCl) methanol and maintained at 4°C for 24 h. The absorption of anthocyanins at 550nm was measured by a spectrophotometer. Concentration of anthocyanins was determined by using the extinction coefficient [17]:

$$\varepsilon_{550} = 33{,}000\ (cm^2/moll.)$$

The experiment was carried out as a factorial experiment based on a completely randomized design (CRD) with two factors and 5 replications per treatment and two jars per replication. The first factor was the concentrations of PEG (0, 3, and 6%), and the second was the almond genotypes. Statistical analysis of the data was carried out by SPSS 16.0, SPSS Inc. The results subjected to an analysis of variance (ANOVA) and difference among treatments means were compared by using Duncan's multiple range test at $P\leq0.05$.

III. Results

Table I shows the results of analyses of variance (ANOVA) of the effects of PEG treatments on physiological responses of almond genotypes. Water saturation deficit (WSD) was significantly affected by genotype and drought stress treatments (Table I). WSD was significantly higher in 7.0% PEG treatment (Fig. 1). The highest WSD value was found in 'Mamaei' and the lowest value was found in 'Supernova'.

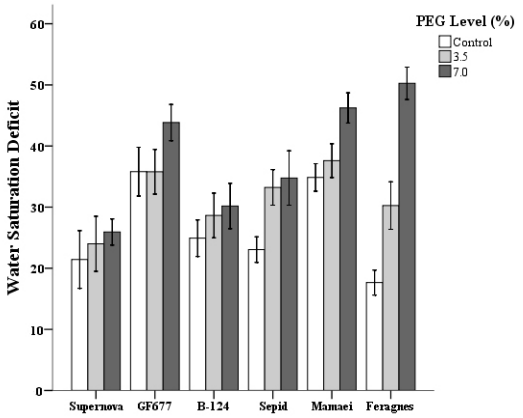

Fig. 1 Effect of osmotic stress on leaf water saturation deficit of almond genotypes

Osmotic stress significantly reduced CMS in the leaves of the almond genotypes (Table I). CMS was significantly lower in the 7.0% PEG treatment. The lowest CMS values were found in the leaves of 'Sepid', 'Mamaei' and 'Ferragnès' under 7.0% PEG treatment. At the end of the experiment, CMS of 'Supernova' and GF677 leaves were maintained at higher level under osmotic stress (Fig. 2).

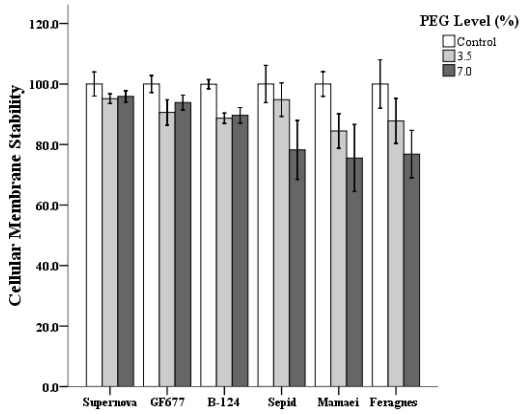

Fig. 2 Effect of osmotic stress on leaf cellular membrane stability of almond genotypes

Effects of osmotic stress treatments and genotype on concentrations of photosynthesis pigments and anthocyanins in the leaves were significant (Table I). Photosynthesis pigments were significantly reduced by osmotic stress (Fig. 3). Total chlorophylls contents as well as chlorophyll a:b ratio were significantly higher in the leaves of 'Supernova' and GF677.

TABLE I
ANOVA Results of Experimental Factors on Physiological Responses of Almond Genotypes

Source of Variations	*df*	WSD	CMS	Total Chl.	Chl. *a* : Chl. *b*	Chls : Carotenoids	Anthocyanins
Genotype	5	415.45**	172.02 ^{ns}	3009.51**	0.389*	0.79**	0.24**
Drought Stress	2	785.58**	1407.56**	33490.88**	31.81**	8.96**	0.22**
Genotype × Drought Stress	10	110.38 ^{ns}	107.77 ^{ns}	481.92 ^{ns}	0.019 ^{ns}	0.098 ^{ns}	0.04 ^{ns}
Error	72	80.76	112.01	857.81	0.13	0.23	0.01

**. significant at $P \leq 0.01$; ns. Non-significant.

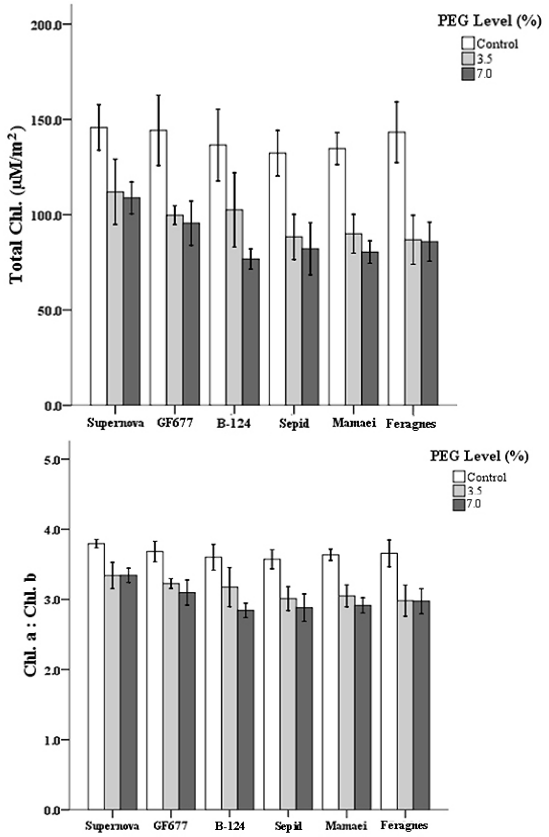

Fig. 3 Effect of osmotic stress on concentration of photosynthesis pigments in the leaves of almond genotypes

The ratio of chlorophylls to carotenoids showed significant reduction under osmotic stress conditions. Chlorophylls to carotenoids ratio was significantly higher in the leaves of GF677 and 'Supernova' (Fig. 4).

Concentration of anthocyanins in the leaves of the almond genotypes was significantly reduced by increasing PEG level in the media. Leaf anthocyanins concentration was significantly higher in 'Supernova', and the lowest anthocyanins concentration was found in the leaves of 'Ferragnès' (Fig. 5).

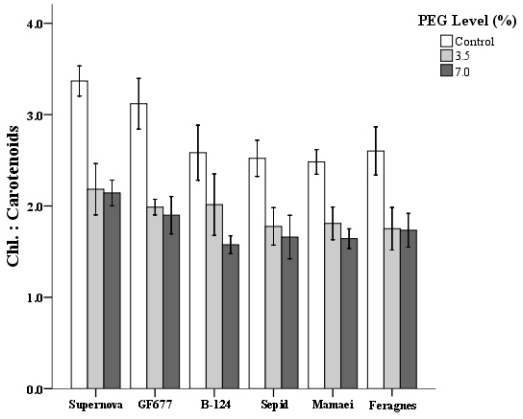

Fig. 4 Effect of osmotic stress on concentration of carotenoids in the leaves of almond genotypes

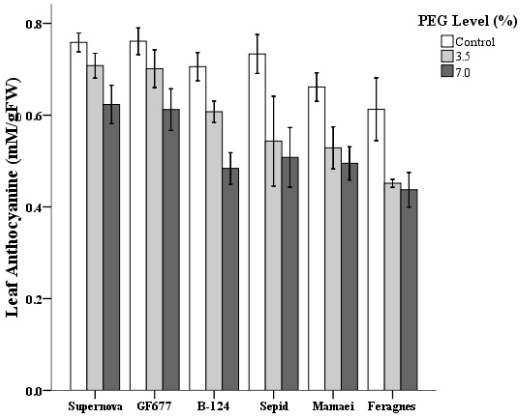

Fig. 5 Effect of osmotic stress on concentration of anthocyanins in the leaves of almond genotype

IV. Discussion

In this study, CMS decline in the leaves of almonds was in coincidence with increased water saturation deficit (WSD) and cell dehydration under drought stress (Figs. 1 and 2). Sivritepe et al. [18], and Karimi et al. [5] also reported drought stress and cell dehydration cause damages to the cell structures. Bajji et al. [19] have also showed that decline of CMS is correlated with water content reduction of tissues. Water limitation and cell dehydration bring about some malfunctions of cell metabolism which lead to reactive oxygen species (ROS) formation. ROS damaged cell membrane and other cell structures which resulted in CMS decline. Decrease in chlorophyll concentration and yellowing of the almond genotypes' leaves may be referred to as visual symptoms of extreme cellular damages under severe drought stress.

There are many reports on chlorophyll decline under drought stress. Structural damages to chloroplasts due to elevated ROS formation and/or photo degradation of the pigments probably leads to loss of chlorophylls in the leaves under drought stress [20]. Lower level of chlorophyll a:b ratio under PEG treatments indicates the constructional damage of chloroplasts whit WSD increase. Chlorophyll concentration in the leaves of almonds were reduced under drought stress. However, it remained higher in the leaves of 'Supernova' and GF677. Maintaining chlorophylls under drought stress is a drought tolerance trait which can be used in screening drought tolerant genotypes [21].

The results revealed that concentration of carotenoids remains higher in the leaves of drought tolerant almond genotypes under osmotic stress conditions. Carotenoids have a critical role in photoperotection by quenching triplet chlorophyll and singlet oxygen derived from excess light energy, thus may limit structural damages under water stress. Carothenoids are also responsible for the scavenging of singlet oxygen [22], thus maintaining carotenoids in the leaves of the tolerant cultivars, 'Supernova' and GF677, may explain less structural damage and higher chlorophyll concentration in their leaves. The higher ratio of chlorophyll to carotenoids

indicates the capacity of higher carotenoid concentration to protect the photosynthetic apparatus [23].

Anthocyanins were significantly reduced under osmotic stress. However, their concentration remained higher in the leaves of the drought tolerant genotypes, 'Supernova' and GF677. Complementary to their photoprotective function, anthocyanins have also demonstrated potent antioxidant capabilities [24]. Neill [17] showed that anthocyanins could provide widespread cellular protection for cellular membranes, organelles, and DNA. Close and Close et al. [25] have reviewed and discussed that anthocyanins act as compatible solutes in osmotic regulation, too. Hence, preserving anthocyanins at higher level in the leaves of 'Supernova' and GF677 may be considered as another protective mechanism against drought stress.

V. Conclusion

The data suggested the possibility of screening drought tolerance in almond genotypes using *in vitro* experiments. Water saturation deficit and cellular membrane stability were found reliable parameters to screen drought tolerance in almond. Preserving higher concentrations of anthocyianins and carotenoids in the leaves were found to be drought tolerance related traits. Anthocyianins and carotenoids probably by scavenging of reactive oxygen species and photoprotection mechanisms reduce the destructive effects of drought stress on almond.

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